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13. SUPPLEMENTARY NOTES

14. ABSTRACT:

Transforming growth factor beta (TGFB) signaling regulates key reproductive events via TGFBR1/TGFBR2. To determine potential effect of overactivation of TGFB signaling in the oocyte, we generated a mouse model containing a constitutively active TGFBR1 using growth differentiation factor 9 (*Gdf9*)-Cre (i.e., TGFBR1-gCA). Follicle counting demonstrated that the number of primordial, primary, and secondary follicles was reduced in TGFBR1-gCA ovaries compared with controls at P7. Concomitantly, abnormal follicle structures were detected in TGFBR1-gCA ovaries, evidenced by INHA staining. These results suggest that sustained activation of TGFBR1 using *Gdf9*-Cre disrupts folliculogenesis via affecting ovarian reserve and follicle growth/development. Apoptosis analysis using ovaries at critical timepoints during follicular development did not reveal alteration of oocyte apoptosis in TGFBR1-gCA ovaries. Immunostaining was performed to determine the molecular identify of the tumors. The results showed that ovarian tumor tissues from TGFBR1-gCA mice were positive for granulosa cell markers FOXL2, INHA, and FOXO1, supporting the formation of granulosa cell tumors in these mice. In the ovary culture system, SB-505124 seemed to improve follicle development in TGFBR1-gCA ovaries. Therefore, sustained activation of TGFBR1 using *Gdf9*-Cre leads to the development of ovarian neoplasms reminiscent of granulosa cell tumors.

15. SUBJECT TERMS
Ovarian tumor, TGF-beta signaling, Follicular development, Proliferation

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1. Introduction

Transforming growth factor beta (TGFB) signaling regulates multiple cellular functions and key reproductive events via transmembrane receptors (TGFBR1/TGFBR2) and intracellular mediators of SMAD proteins. Until now, the function of TGFB signaling in mouse oocytes remains elusive. The aim of this project during the reporting period is to define the oncogenic role of constitutively active TGFBR1 in the oocyte using *Gdf9*-Cre and the role of sustained activation of oocyte TGFBR1 in ovarian tumor development using an *in vitro* approach.

2. Keywords

Ovarian tumor, Sex cord-stromal tumor, TGF-beta signaling, Overactivation, *Gdf9*-Cre, Follicular development, Proliferation, Apoptosis, Malignant transformation

3. Accomplishments

- *What were the major goals of the project?*

The major goal during this reporting period is to define the oncogenic role of constitutively active TGFBR1 in the oocyte using *Gdf9*-Cre and the role of sustained activation of oocyte TGFBR1 in ovarian tumor development using an *in vitro* approach. The milestones are: 1.) The effect of sustained activation of TGFBR1 in the oocyte identified (8 months), and 2.) The role of sustained activation of TGFBR1 in the oocyte *in vitro* identified (4 months). These milestones have been achieved during this period.

- *What was accomplished under these goals?*

1.) Major activities and specific objectives

A. We collected postnatal ovaries from control and TGFBR1-gCA females at postnatal days and performed histological assays and follicle number analysis. The specific objective of these studies is to determine the effect of sustained activation of TGFBR1 using *Gdf9*-Cre on primordial follicle activation.

B. We performed apoptosis analysis using ovaries from both control and TGFBR1-gCA mice at P3, P7, P12, and P21. The specific objective is to determine whether oocyte death and apoptosis is a potential contributing factor to granulosa cell tumor development.

C. We performed immunohistochemistry analysis of ovaries from control and TGFBR1-gCA mice at the age of 8 weeks. We used multiple granulosa cell markers including alpha inhibin (INHA), forkhead box protein O1 (FOXO1), forkhead box L2 (FOXL2), and anti-Mullerian hormone (AMH) and germ cell marker DEAD-box helicase 4 (DDX4). SRY-box 9 (SOX9), a Sertoli cell marker was also analyzed. The specific objective of these studies is to determine the molecular type of ovarian tumors in our model.

D. We collected ovaries and isolated oocyte cumulus complexes from control and TGFBR1-gCA mice. We performed ovary culture in the absence or presence of TGFBR1 inhibitor, SB 505124. The effect of TGFBR1 inhibition on follicular development was analyzed.

2.) Significant results or key outcomes

A. Overactivation of TGFBR1 using *Gdf9*-Cre perturbed follicular development at an early stage. Because *Gdf9*-Cre is expressed in primordial follicles, we performed histological analysis of follicular development in control and TGFBR1-gCA mice at early postnatal stages. To perform follicle counting, ovaries were first serially sectioned (5 μm) and stained with periodic acid Schiff's (PAS) and hematoxylin. Follicles were counted from every 5th section. Follicle classification was based on morphological criteria (Myers *et al.* 2004, Bristol-Gould *et al.* 2006). Morphologically, a primordial follicle contains an oocyte that is partially or completely encapsulated by one layer of squamous somatic cells, whereas a primary follicle has an oocyte surrounded by one layer of cuboidal granulosa cells. Follicles at transitional stages may contain both squamous and cuboidal granulosa cells, and were defined as primary follicles if surrounding somatic cells were mainly cuboidal. A secondary follicle consists of 2 or more layers of granulosa cells but has no antrum. Primordial and primary follicles were counted, regardless of the status of the oocyte nucleus. Since the oocyte diameter for primordial and primary follicles averages ~ 14 μm, the same follicle could be counted every two sections. Hence, the estimated primordial and primary follicle numbers per ovary were calculated by multiplying the cumulative number of counts of each ovary by 5 and dividing by 2. For secondary follicles, only oocytes with nucleus were counted and a correction factor of 5 was applied to estimate the total number of secondary follicles per ovary (Bristol-Gould *et al.* 2006, Ren *et al.* 2012).

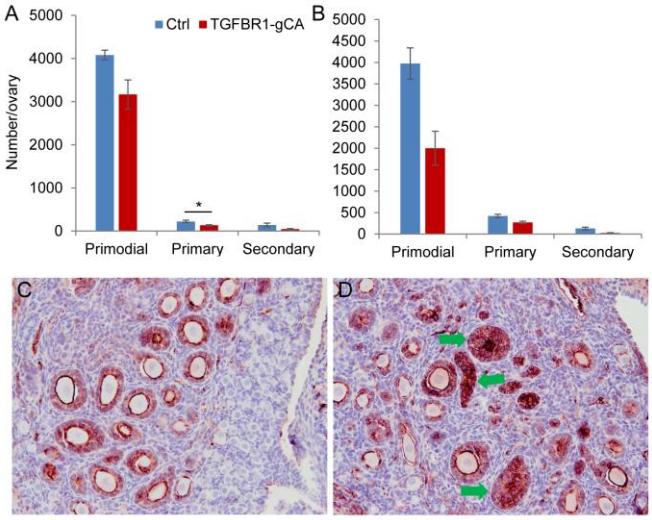

Figure 1. Altered follicular development in TGFBR1-gCA mice. (**A** & **B**) Follicle counts of control and TGFBR1-gCA ovaries at P5 (A) and P7 (B). Follicles were counted from every 5th section. Data are mean ± s.e.m. n = 3. $^{*}P < 0.05$. (**C** & **D**) Immunohistochemical localization of INHA in P7 control (C) and TGFBR1-gCA (D) ovaries. Arrows (D) indicate abnormal follicle structures. Experiment was performed using ABC method, and signals were developed using NovaRED Peroxidase Substrate Kit. Magnification: 200x.

Follicle quantification did not show a significant difference in primordial follicles between control and TGFBR1-gCA mice at postnatal day 5 (P5), although there was a reduction of primary follicle numbers in the TGFBR1-gCA mice versus controls (Figure 1A). At P7, the number of primordial follicles, primary follicles, and secondary follicles was reduced in TGFBR1-gCA ovaries compared with controls (Figure 1B). At this stage, abnormal follicle structures were detected in the ovary of TGFBR1-gCA mice, evidenced by INHA staining (Figure 1C and D). These results suggest that

sustained activation of TGFBR1 in the oocytes using *Gdf9*-Cre disrupts ovarian folliculogenesis by affecting ovarian reserve and subsequent follicle development.

B. Activation of TGFBR1 using *Gdf9*-Cre did not promote apoptosis in the oocyte. It has been suggested that loss of oocytes during follicular development may alter the differentiation and cell fate of ovarian granulosa cells (Pitman *et al.* 2012). We therefore performed apoptosis analysis to determine whether there is a link between oocyte apoptosis and abnormal granulosa cell differentiation and tumor formation. To achieve this goal, we utilized a commercially available *In situ* Apoptosis Detection Kit to stain ovarian samples at P3, P7, P12, and P21 (n = 5). Briefly, paraffin sections were deparaffinzed in xylene and rehydrated in graded alcohol before treated with Proteinase K. Then 3% H_2O_2 was applied to inactivate endogenous peroxidases. Apoptotic cells were labeled with terminal deoxynucleotidyl transferase (TdT) and incubated with streptavidin-horseradish peroxidase (HRP) conjugate. The signals were detected using diaminobenzidine (DAB) and slides were counterstained with Methyl Green. To validate this kit, we showed that samples treated with DNase I contained abundant apoptotic cells (i.e., positive control), whereas negative controls where TdT was substituted with water showed only background staining (Figure 2A and B). Our apoptosis analysis using ovarian samples from several critical time points during early follicular development did not reveal alteration of oocyte apoptosis in TGFBR1-gCA ovaries (Figure 2C and D). Extensive apoptosis was detected in ovarian somatic cells at P21 in TGFBR1-gCA mice compared with controls, where apoptosis was confined to certain follicles that were atretic or would potentially be atretic (Figure 2E and F). Thus, these results suggest that oocyte apoptosis may not be a main contributing factor to ovarian tumor development in our mouse model.

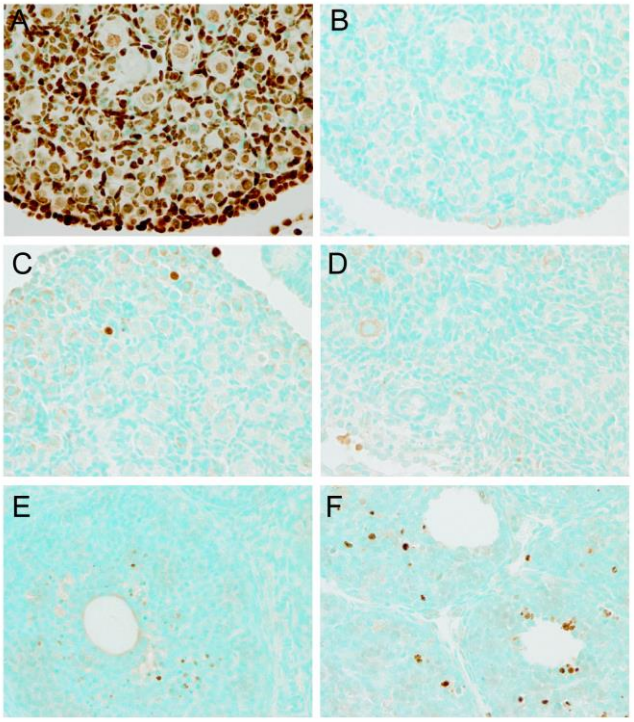

Figure 2. Apoptosis analysis of postnatal ovaries from control and TGFBR1-gCA mice. (**A**) P3 ovarian sections treated with DNase I as positive control. (**B**) Negative control where TdT was replaced with water. (**C-F**) Representative images of apoptosis analysis of P3 and P21 control (C and E) and TGFBR1-gCA (D and F) ovarian sections. Apoptotic cells were labeled with terminal deoxynucleotidyl transferase (TdT) and signals were detected using DAB. Sections were counterstained with Methyl Green. At least 4 independent samples per group were used in this analysis. Magnification: 400x.

C. Molecular analysis of ovarian tumor type. To define the molecular identity of ovarian tumors in TGFBR1-gCA mice, we performed immunostaining to determine expression of a granulosa cell lineage maker FOXL2 (Schmidt *et al.* 2004) and 3 other granulosa cell-expressed proteins, FOXO1, INHA, and AMH. DDX4, a germ cell marker, was also included. Briefly, Tissue processing and embedding were carried out using the histology core facility of the Department of Veterinary Integrative Biosciences at

Texas A&M University. Paraffin sections (5 μm) were used for both immunofluorescence and immunohistochemistry as described (Li *et al.* 2011, Gao *et al.* 2014). Briefly, sections were deparaffinized in xylene and rehydrated in graded alcohol. Then the slides were subject to antigen retrieval using citrate buffer. For immunohistochemical analysis, sections following antigen retrieval were treated with H_2O_2 and blocked with 5% non-immune serum, and incubated with primary and secondary antibodies and Avidin/Biotin Complex (ABC; Vector Laboratories). The signals were developed using NovaRED Peroxidase Substrate Kit (Vector Laboratories). The sections were counterstained with hematoxylin and mounted with Permount. For immunofluorescence microscopy, the sections were blocked with 5% bovine serum albumin (BSA) and sequentially incubated with primary antibodies and secondary antibodies conjugated with Alexa Fluor 594 (1:400; Invitrogen). The slides were mounted using ProLong Gold Slowfade media with DAPI. The immunofluorescence signals were examined using an IX73 fluorescence microscope. Images were captured using an XM10 CCD camera and cellSens® Digital Imaging Software (Olympus). Isotype-matched IgGs were included as negative controls for these assays.

The localization of FOXL2 (Figure 3A), INHA (Figure 3C), FOXO1 (Figure 3E), AMH (Figure 3G), and DDX4 (Figure 3I) was detected in the granulosa cell or oocyte compartment of the control mice, while ovarian tumor tissues from TGFBR1-gCA mice were immunoreactive with FOXL2 (Figure 3B), INHA (Figure 3D), and FOXO1 (Figure 3F), supporting the development of granulosa cell tumors in these mice. However, expression of AMH was close to background level in the tumor tissues (Figure 3H). These tumors did not express DDX4 (Figure 3J). Representative negative controls using rabbit and goat IgGs were respectively shown in

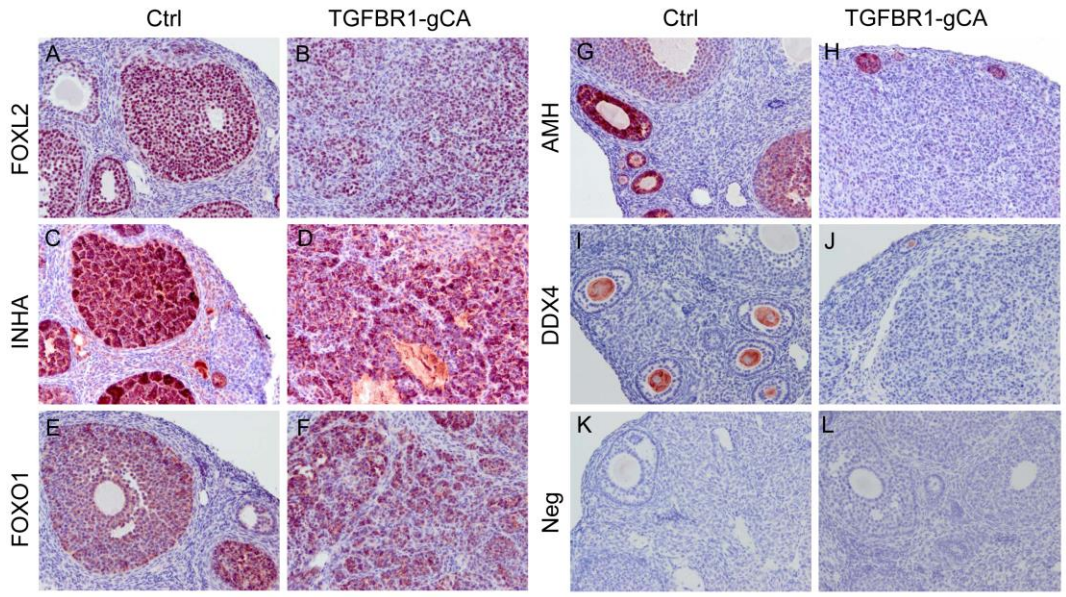

Figure 3. Identification of tumor type using molecular markers. (**A-J**) Expression of granulosa cell and germ cell markers in control and TGFBR1-gCA ovaries. Representative images from immunohistochemical analysis of FOXL2 (A and B), INHA (C and D), FOXO1 (E and F), AMH (G and H), and DDX4 (I and J) are shown. (**K** & **L**) Negative controls using isotope-matched rabbit (K) and goat (L) IgGs. Mice at the age of 8 weeks were utilized for this experiment. Experiment was performed using ABC method, and signals were developed using NovaRED Peroxidase Substrate Kit. n = 5. Magnification: 200x.

Figure 3K and L. Because granulosa cell tumors can express Sertoli cell marker (Liu *et al.* 2015), we examined whether Sertoli cell-like components were present in the TGFBR1-gCA tumors. Our results showed expression of SOX9 in ovarian tumors resulting from TGFBR1 overactivation (Figure 4D-I; arrows). Thus, sustained activation of TGFBR1 using *Gdf9*-Cre causes the formation of ovarian neoplasms reminiscent of granulosa cell tumors.

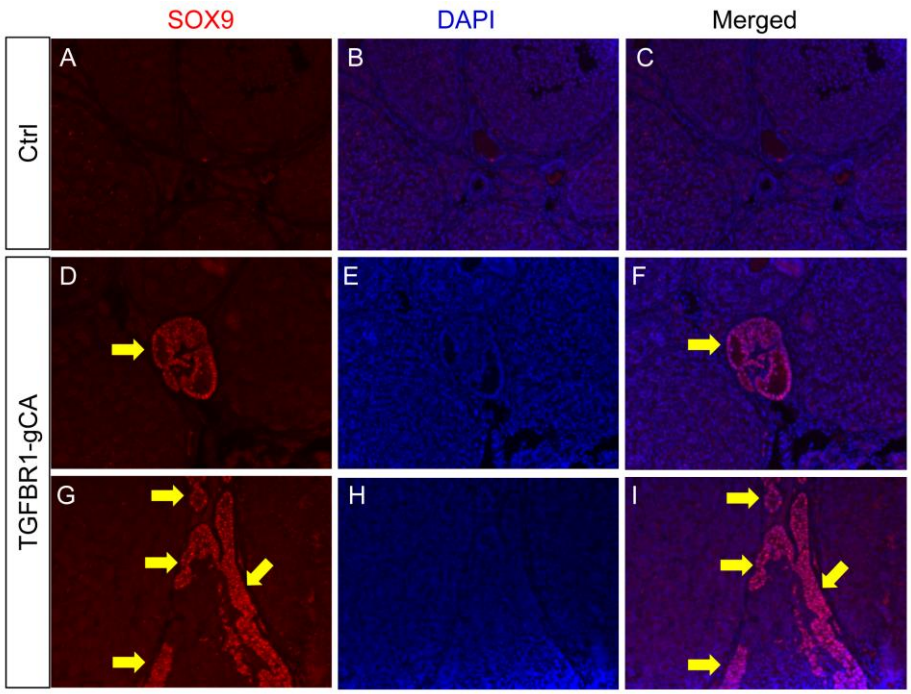

Figure 4. Immunofluorescence of SOX9 in control and TGFBR1-gCA ovaries. (**A-C**) SOX9 expression in control ovaries. (**D-I**) SOX9 expression in TGFBR1-gCA ovaries. DAPI (blue) was used to counterstain the nucleus. Two independent TGFBR1-gCA mice (D-F and G-I) were representatively shown. Arrows indicate abnormal expression of SOX9 proteins (red) in ovarian tumor tissues. Three control and TGFBR1-gCA mice at the age of 8 weeks were analyzed. Magnification: 200x.

D. Identify the role of sustained activation of oocyte TGFBR1 in ovarian tumor development using *in vitro* approach. We took advantage of a small molecule inhibitor of TGFBR1 to further elucidate the oncogenic property of the constitutively active TGFBR1 using *in vitro* culture approach. In brief, we collected ovaries from control and TGFBR1-gCA mice and cultured them using Transwell membrane insert (Dutta *et al.* 2014). Ovaries were cultured in the presence or absence of SB-505124 for 10 days at 37°C supplemented with 5% CO2. Culture was fed every 2 days. After that, the ovaries were fixed and processed for histological analysis. We also isolated oocyte-granulosa cell complex (OGC) by using 0.1% collagenase digestion of P12 ovaries (Eppig & O'Brien 1996). While OGCs retrieved from the control mice were morphologically normal and similar in size, OGCs obtained from the TGFBR1-gCA mice varied in size and contained large abnormal structures, suggesting that overactivation of TGFBR1 using *Gdf9*-Cre disrupts folliculogenesis and promotes the formation of abnormal OGCs. Interestingly, our culture experiment revealed disorganized and abnormal follicle structures within vehicle-treated TGFBR1-gCA ovaries (Figure 5A and B). Incubation of TGFBR1-gCA ovaries with SB-

505124 (10 µM) seemed to improve follicle development in the TGFBR1-gCA ovaries (Figure 5C and D). Therefore, these studies reinforce that ovarian tumor development in TGFBR1-gCA mice is due to enhanced TGFBR1 activity.

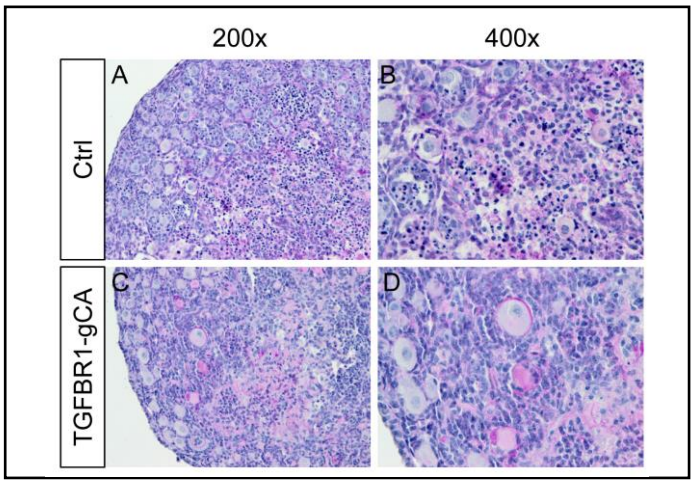

Figure 5. Histological analysis of cultured TGFBR1-gCA ovaries treated with vehicle and TGFBR1 inhibitor. (**A** & **B**) TGFBR1-gCA ovaries treated with vehicle control. (**C** & **D**) TGFBR1-gCA ovaries treated with SB-505124. Ovary culture was performed using Transwell membrane insert in the presence or absence of SB-505124 (10 µM) for 10 days before histological analysis using periodic acid Schiff's (PAS) and hematoxylin staining. Magnification: 200x (A & C) and 400x (B & D).

- What opportunities for training and professional development has the project provided?

  Nothing to Report.

- How were the results disseminated to communities of interest?

  Nothing to Report.

- What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, we plan to continue the research activities described in the original proposal. Two major goals are: 1.) Explore the stage-specific function of constitutively active TGFBR1 in the oocyte; and 2.) Discover novel regulatory mechanisms of granulosa cell proliferation resulting from constitutive activation of TGFBR1 in the oocyte. We will create a new mouse model using *Zp3*-Cre to determine whether the effect of TGFBR1 activation is follicle stage dependent. We will also perform RNA sequencing analysis to understand the mechanism of the ovarian tumor phenotype in our model system.

4. Impact

- What was the impact on the development of the principal discipline(s) of the project?

The potential impact of this project is to identify new diagnostic and therapeutic targets for ovarian granulosa cell tumors.

- What was the impact on other disciplines?

  Nothing to Report.

- What was the impact on technology transfer?

Nothing to Report.

- What was the impact on society beyond science and technology?

  Nothing to Report.

5. Changes/Problems

- Changes in approach and reasons for change

  Nothing to Report.

- Actual or anticipated problems or delays and actions or plans to resolve them

  Nothing to Report.

- Changes that had a significant impact on expenditures

  Nothing to Report.

- Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

  Nothing to Report.

6. Products

- Publications, conference papers, and presentations

  Yang Gao and Qinglei Li. Sustained activation of transforming growth factor beta signaling using growth differentiation factor 9-driven iCre disrupts postnatal folliculogenesis and promotes malignant transformation of ovarian somatic cells (**Poster presentation**). *22nd Annual Meeting, Texas Forum for Reproductive Sciences*, Houston, Texas, USA, April 21 & 22, 2016.

- Website(s) or other Internet site(s)

  Nothing to Report.

- Technologies or techniques

  Nothing to Report.

- Inventions, patent applications, and/or licenses

  Nothing to Report.

- Other Products

  This experiment created a mouse model that develops ovarian tumors (i.e., TGFBR1-gCA), which could be potentially used for preclinical testing of therapeutic agents.

7. Participants & Other Collaborating Organizations

- What individuals have worked on the project?

Name	Project Role	Nearest person month worked	Contribution to the project
Yang Gao	Graduate Student	6 Calendar	Contributed to sample collection, histological analysis, oocyte granulosa cell complex isolation, immunostaining, apoptosis detection, and data analysis
Qinglei Li	PI	3 Calendar	Contributed to sample collection, data analysis, and project supervision
David Threadgill	Co-investigator	0.6 Calendar	Contributed to experimental design
Robert Burghardt	Co-investigator	0.6 Calendar	Contributed to ovarian tissue culture

- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

  Nothing to report.

- What other organizations were involved as partners?

  Nothing to report.

8. Special Reporting Requirements

None.

9. Appendices

References Cited under *Significant results or key outcomes*

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